EFFECTS OF BASIDIOMYCETE LACCASE ON CERCOSPORIN

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SUMMARY

Cercosporin is a perylenequinone pigment produced by fungi in the genus *Cercospora* which under light generates reactive oxygen species causing membrane damage and mortality of living cells. Our objectives were to demonstrate that fungal laccase, a lignolytic copper-containing enzyme, can degrade cercosporin and reduce cercosporin toxicity toward living cells. Cercosporin from *Cercospora beticola* and *Cercospora hayi* was treated with laccase from basidiomycete fungi (*Pleurotus ostreatus* and *Trametes versicolor*) in the dark and under constant light. Under these conditions the absorbance of the cercosporin decreased at 220, 279 and 295-500 nm within 10 min of reaction with laccase from either *P. ostreatus* or *T. versicolor*, indicating that basidiomycete laccase can induce changes in UV-visible spectra of cercosporin. The LIVE/DEAD® Bac Light™ VIABILITY kit and fluorescent microscopy showed more viable *E. coli* cells after incubation under light with cercosporin and laccase than with cercosporin alone. Lesions were apparent on sugar beet leaves exposed to cercosporin under light after 48 h, but leaves exposed to cercosporin and laccase showed visibly less damage. These data suggest that laccase from basidiomycete fungi can decrease the toxic effect of cercosporin toward microorganisms and plant tissue.

INTRODUCTION

Cercosporin (Kuyama and Tamura, 1957; Lynch and Geoghegan, 1979) is a perylenequinone pigment (Fig. 1) produced by fungi in the genus *Cercospora*, that belongs to a class of molecules called photosensitizers. Photosensitizers are characterized by the ability to be activated by light and to react with oxygen to produce highly toxic reactive oxygen species, such as superoxide anions ($O.^-_2$) and singlet oxygen ($^1O_2$) (Daub, 1982; Daub and Hangarter, 1983). These reactive molecules cause cell death of several organisms including mice, fungi and bacteria (Yamazaki *et al.*, 1975; Macri and Vianello, 1979; Hartman *et al.*, 1988; Daub *et al.*, 1992).

Cercospora species appear to resist high concentrations of reactive oxygen species. Although the mechanisms providing cercosporin resistance to *Cercospora* is not fully understood yet, reports have demonstrated that *Cercospora* species were cercosporin-resistant organisms capable of protecting themselves against cercosporin and the damage of reactive oxygen species by the reduction and detoxification of cercosporin into an inactive form lacking photodynamic activity (Daub *et al.*, 1992). Jenns *et al.* (1995) demonstrated that cercosporin-sensitive mutants of *Cercospora nicotianae* are unable to reduce cercosporin. Also, it has been reported that a protein similar to pyridine nucleotide reductase in the yeast *Saccharomyces cerevisiae* (Ververidis *et al.*, 2001; Panagiotis *et al.*, 2007) or an oxidoreductase produced by the bacterium *Xanthomonas campestris* pv. zinniae, can be used for cercosporin detoxification (Taylor *et al.*, 2006).

Many fungi contain enzymes such as superoxide dismutase, peroxidases, catalase, and perhaps laccases and

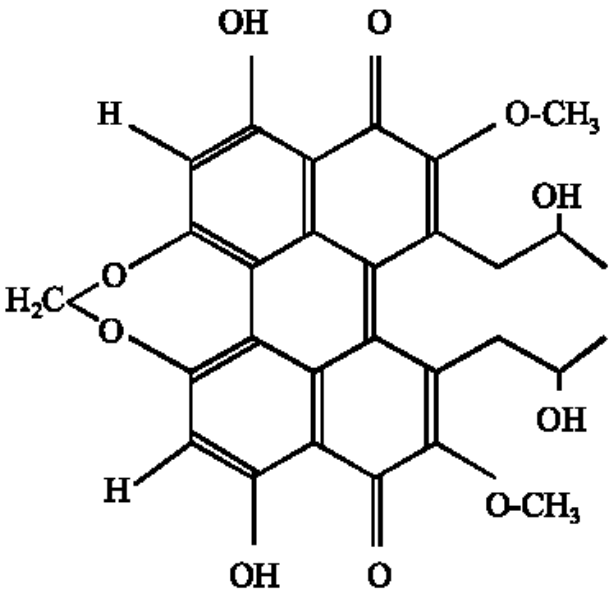

Fig. 1. Chemical structure of cercosporin.

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polyphenol oxidases that can help to remove or inactivate reactive oxygen species (Mayer *et al.*, 2001). Laccase is a phenol oxidoreductase containing copper atoms which can oxidize organic and inorganic substrates, including both phenolic and non-phenolic compounds, to their corresponding quinones, with the concurrent reduction of molecular oxygen and release of water (Leonowicz *et al.*, 1979; Thurston, 1994). Although little is yet known in the literature about the exact mechanisms by which laccases interact with reactive oxygen species in general, it was speculated that an intermediate reaction product of the activity of laccases, which is a free radical, could undergo non-enzymatic reactions with a variety of these damaging oxygen species (Ferrari *et al.*, 1997; Duran and Esposito, 2000; Claus, 2004). It has been reported that white rot basidiomycete fungi (wood-decaying basidiomycetes) increase their extracellular discharge of laccase to detoxify their environment under culture conditions as a defense mechanism against oxidative stress conditioning factors, such as H_2O_2, (Cho *et al.*, 2006) or paraquat which catalyzes the overproduction of superoxide anion radicals ($O.^-_2$) and consequently other oxygen active species (Jaszek *et al.*, 2006). Laccase has been proposed for a diverse range of applications, including waste detoxification and textile dye transformation (Rama *et al.*, 1998, Abadulla *et al.*, 2000; Fukuda *et al.*, 2001), degradation of lignin (Bourbonnais *et al.*, 1995) and humic acids (Hofrichter and Fritche, 1997) but little is known about the effects of laccase on cercosporin. In this preliminary study, we propose that laccase from basidiomycete fungi can decrease the toxicity of cercosporin toward living cells.

The first objective of this study was to measure the time evolution of the absorption spectrum of cercosporin from *Cercospora beticola* Sacc. C2 isolate and *C. hayi* L. Calpouzos with or without addition of laccase from two white-rot basidiomycete fungi, *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. and *Trametes versicolor* (L.:Fr.) Pilát. We wanted to investigate the effects of cercosporin and/or laccase on living organisms, including bacteria and fresh sugar beet leaves. Although fungicides and appropriate cultural practices such as tillage and crop rotation (Ward *et al.*, 1999; Windels *et al.*, 1998) are successfully used to control diseases caused by *Cerco-*

Table 1. Viability of *E.coli* cells in *Cercospora beticola* (isolate C2)-containing medium with or without *P. ostreatus* laccase added, in the dark and under constant light.

		Number of viable cells (%)		
Incubation conditions	Treatments*	1h**	2h	3h
Dark	Cercorporin	96.51 A	96.40 A	96.31 A
	Cercosporin + laccase	97.20 A	96.78 A	96.73 A
	Laccase	97.28 A	97.51 A	97.21 A
	Control	96.51 A	97.75 A	97.59 A
Light	Cercorporin	38.79 B	54.44 C	53.28 C
	Cercosporin + laccase	89.07 A	88.86 B	90.56 B
	Laccase	95.32 A	95.99 A	96.04 A
	Control	97.06 A	97.67 A	97.22 A

*Controls contain neither cercosporin nor laccase; cercosporin and laccase concentration in LB medium are 100 _g mL^{-1} and 100U ml^{-1} respectively;

** incubation time of bacteria after treatment.

Numbers followed by different letters within a column are significantly different by the Tukey-Kramer Honesty Significance Difference mean separation at *P ≤ 0.05.

Table 2. Effects of *P. ostreatus* laccase and cercosporin from *Cercospora beticola* (isolate C2) sugar beet leaf tissue (in a scale of 1-5) after 48 h of incubation in the dark and under constant light.

Treatments	Dark	Light
Cercosporin (20)*	2	5
Cercosporin + laccase (24)	2	2
Laccase (20)	2	2
Control (20)	1	1

Data were ranked on a visual basis with a scale of 1-5 to describe symptoms at the margins of the treated zones (1) no lesion; (2) dark brown lesions; (3) circular brown-yellow lesions; (4) circular light-yellow necrotic lesions (5) extended light-yellow, dry and crusty necotic lesions.

*Number of treated zones used for ranking.

spora species, alternate methods of control would be desirable. This study seeks to develop insight for a possible approach to target cercosporin by antagonizing *C. beticola* with laccase-producing basidiomycete fungi to decrease *Cercospora* infection.

MATERIALS AND METHODS

Strains, media and culture conditions. *Cercospora beticola* isolate C2 (Whitney and Lewellen, 1976) was obtained from John J. Weiland, USDA, Fargo, ND. Prior to use, the fungus was purified by single conidia isolation. The isolate was maintained on solid potato dextrose agar (PDA, DIFCO, Detroit, MI) at 23ºC with a 5 h photoperiod under fluorescent light (Universal/Hi-vision F32T8/TL735 tubes, Philips, NY, USA, light intensity 7.435±0.064 Rad [watt m^{-2}]) for maximun production of cercosporin. Long-term stocks were maintained on PDA slants stored at 4ºC.

Cercosporin extraction. Cercosporin from *C. beticola* isolate C2 was extracted following a modified procedure of Daub (1982). Briefly, potato dextrose agar (PDA) containing the fungal mycelia (approximately 6 cm of diameter) was removed from the dish and placed on plastic screens in a laminar flow hood to allow the mycelial mat to dry for 2-3 days. Ten mycelial mats (ca 10 g dry weight) were usually extracted at a time. Dried cultures were finely ground in a spice blender for 30 sec. Then materials were extracted in 300 ml ethyl acetate (CCI, USA) with agitation for 1 h. After decanting the solvent, the procedure was repeated four times until no further red pigment was extracted from the mycelia. The red pigment formed in culture is indicative of cercosporin production (Daub and Ehrenshaft, 2000). The extract was centrifuged at 500 g (Sorvall, USA) for 10 min at 4°C and any remaining solvent was removed using a rotary evaporator (Brinkman Buchii RE111, Switzerland). The residue was dissolved with 0.5 ml chloroform and 1 ml of pentane (Sigma-Aldrich, USA) was added to the mixture. The extract was protected from light and placed in the freezer for at least one week until formation of crystals. The crystallization process (the steps described above beginning with resuspension in ethyl acetate), was repeated 2 additional times to purify the cercosporin. All chemicals were HPLC-grade purity. A commercially-sourced cercosporin extracted from *C. hayi* (Sigma-Aldrich, USA) was also used in this study. Both sources of cercosporin were dissolved in 100% ethanol, filtered through a 0.25 …m pore size acrodisc, and stored in RNase/DNase-free pyrogen tubes at -80°.

Spectrophotometric conditions. Laccase from the basidiomycetes *P. ostreatus* and *T. versicolor* was purchased from a commercial source (Tienzyme Inc. USA). Cercosporin used in the experiment was extracted from *C. beticola* C2 isolate (stock solution: 1 mg ml^{-1}) and from *C. hayi*, the latter commercially purchased (Sigma, USA; stock solution: 3.08 mg ml^{-1}). Laccase (50 U ml^{-1}) from either *P. ostreatus* or *T. versicolor* was added to cercosporin (50 …g ml^{-1}) in MOPS buffer (3-morpholinopropane sulforic acid, 20 mM, pH 6, Sigma-Aldrich, USA) and the mixture was incubated for 0, 2, 4, 6, 8, and 10 min at 25°C in the dark. UV-visible absorption spectrum from 200 to 600 nm was determined using a spectrophotometer (Lamda 20, Perkin-Elmer, USA) with a scanning speed of 960 nm min^{-1}. Controls were cercosporin in buffer without laccase.

To demonstrate that the laccases used in this study had oxidative activity and can react with complex polymeric phenolic compounds other than cercosporin, experiments were conducted using the pigment Poly B-411 dye (Platt *et al.*, 1985) (Sigma-Aldrich, USA) as a substrate, according to the technique of Edens *et al.* (1999). Decolorization of the dye by *P. ostreatus* or *T. versicolor* laccase was monitored at 0, 5, 10, 20, and 30 min, at 25°C by comparing the absorbance ratios 593/483 nm with the controls without laccase added. The assay was performed in triplicate. Experiments were repeated at least three times.

Assays on living microorganisms. Bioassays on bacteria and fresh sugar beet leaves were developed to demonstrate the effects of cercosporin and laccase on living cells. Experiments on bacteria and fresh sugar beet leaves consisted of 4 treatments and 2 incubation conditions. The treatments were a (1) control, (2) cercosporin at 100 …g ml^{-1}, (3) cercosporin at 100 …g ml^{-1} and laccase at 100U ml^{-1}), and (4) laccase at 100 U ml^{-1}). Incubation conditions were in the dark or under constant fluorescent light (as described above in the Materials and Methods). MOPS buffer was used to dilute the chemicals. Only cercosporin from *C. beticola* C2 and *P. ostreatus* laccase were used for the bioassay on bacteria and on fresh leaves.

Bacteria (*E. coli*, TOP10 strain from Invitrogen, USA) were cultured in liquid Luria-Bertani (LB) medium at 37°C for 24 h on a shaker at 200 rpm. After centrifugation at 13,200 g (Eppendorf Centrifuge, model # 5415R, Brinkmann Instruments, USA) for 2 min, bacterial pellets were resuspended in MOPS buffer. Cercosporin and laccase were mixed in MOPS buffer and incubated for 1 h in the dark before adding to the bacterial suspension having a cell concentration of 10^6 cells ml^{-1}. Treated samples and the controls were incubated 1 h on a shaker (200 rpm) at 25°C in the dark or under constant fluorescent light. The LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes, USA) which provides two different nucleic acid stains, the SYTO 9 dye (green fluorescence) and propidium io-

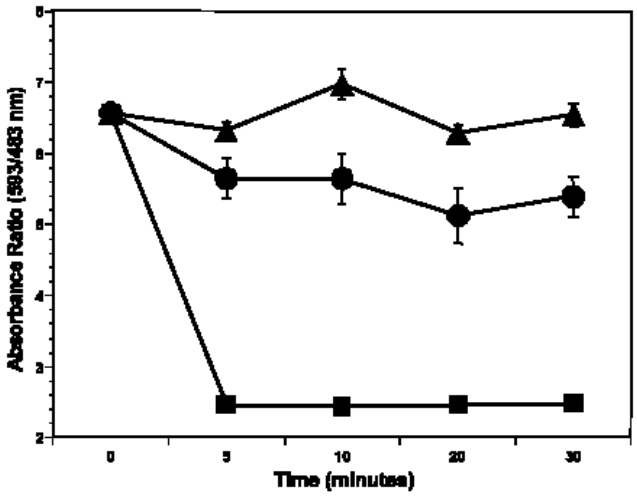

Fig. 2. Decolorization of poly B-411 dye by *P. ostreatus* and *T. versicolor* laccases. Squares indicate reactions in which *Pleurotus ostreatus* laccase was added; circles, reactions where *Trametes versicolor* laccase was added; diamonds, control (no laccase added); results shown are averages of triplicates.

dide (red fluorescence), to rapidly distinguish live bacteria with intact plasma membranes from dead bacteria with compromised membranes respectively, was used according to the manufacturer recommendations. After staining, the cells were rinsed for a few seconds with water then mounted on microscope slides using a medium (Vectashield, Vector Inc., USA) to prevent bleaching of fluorescence. Samples were observed with a confocal scanning laser microscope (Zeiss LSM 410) equipped with a 488nm internal laser and two external lasers (633nm and 543nm) and connected to a Zeiss Axiovert S100 TV. SYTO 9 dye was detected with a 520 nm filter and propidium iodide with a 635 nm filter. A fluorescent oil immersion 100X Plan apochromat (N.A. 1.4) objective was used. Images were scanned to 512 x 512 pixels in each axis. Five images were scanned for each treatment. Pheonix software (Microcosm, USA) was used to count dead and viable cells. Experiments were repeated at least 3 times. The Tukey-Kramer Honesty Significant Difference procedure, standard ANOVA was performed on counts of bacteria with statistical differences evaluated at $P \leq 0.05$ using the JMP statistical software package (version 6.0, 2005, SAS Institute, USA).

Fresh sugar beet (Crystal™ ACH 927 variety) leaves were collected from the field and used to test the effects of cercosporin and/or laccase on plant tissue. Delivery of cercosporin and laccase solutions was performed on sugar beet leaves by lightly touching the surface of the tissue using a syringe (1 ml) and needle (Precision Glide #23G1, Becton Dickinson, NJ, USA). The leaves were placed in a tray layered with wet tissue to prevent desiccation and incubated for 48 h either in the dark or under constant fluorescent light. After incubation, the morphology of the treated areas was assessed visually for each treatment. To rank the various treatments according to the morphology of the lesions, a scale of 0-4 (no lesion to heavy necrotic lesions) was used. Images were captured using a digital camera (Olympus Camedia C-4040 ZOOM). Three to four leaves were used for each treatment and the experiment was repeated twice.

RESULTS AND DISCUSSION

Spectrophotometric studies of laccase-treated cercosporin. Decolorization of pigments or dyes has been used by others as a standard assay to rapidly assess the oxidative activity by ring opening of ligninolytic enzymes of white-rot basidiomycetes, including laccases and peroxidases (Gold *et al.*, 1988, Rodriguez *et al.*, 1999; Claus *et al.*, 2002). In our case, *P. ostreatus* and *T. versicolor* laccases used in this study were able to decolorize

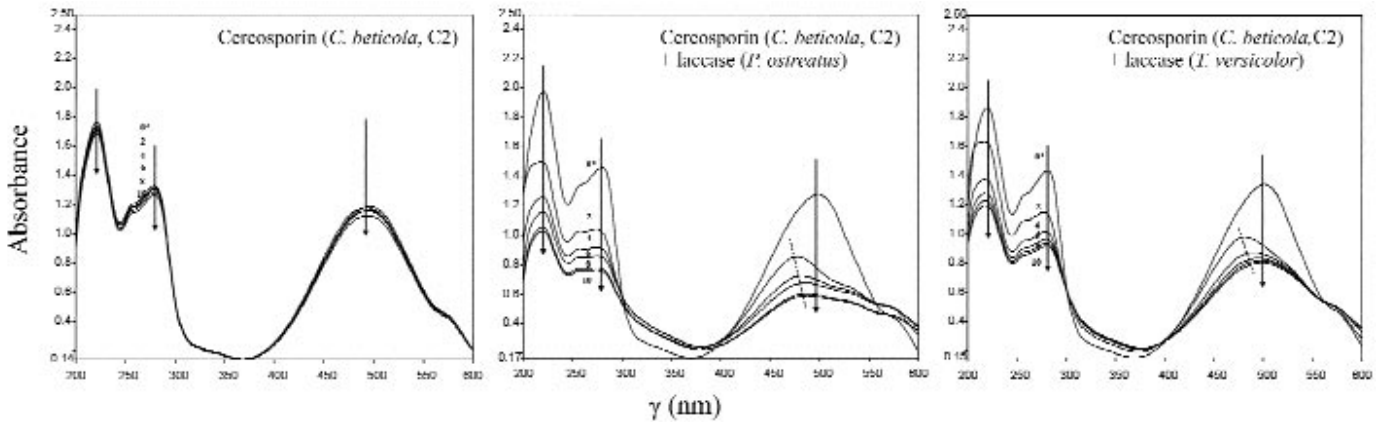

Fig. 3. Effect of *P. ostreatus* and *T. versicolor* laccase on the time evolution of UV-visible absorption spectrum (200-600 nm) of cercosporin from *C. beticola* (isolate C2) in MOPS buffer at 25°C in the dark. Numbers indicate incubation time intervals in minutes; arrows show decrease in absorbance of cercosporin with time. Control is cercosporin without laccase.

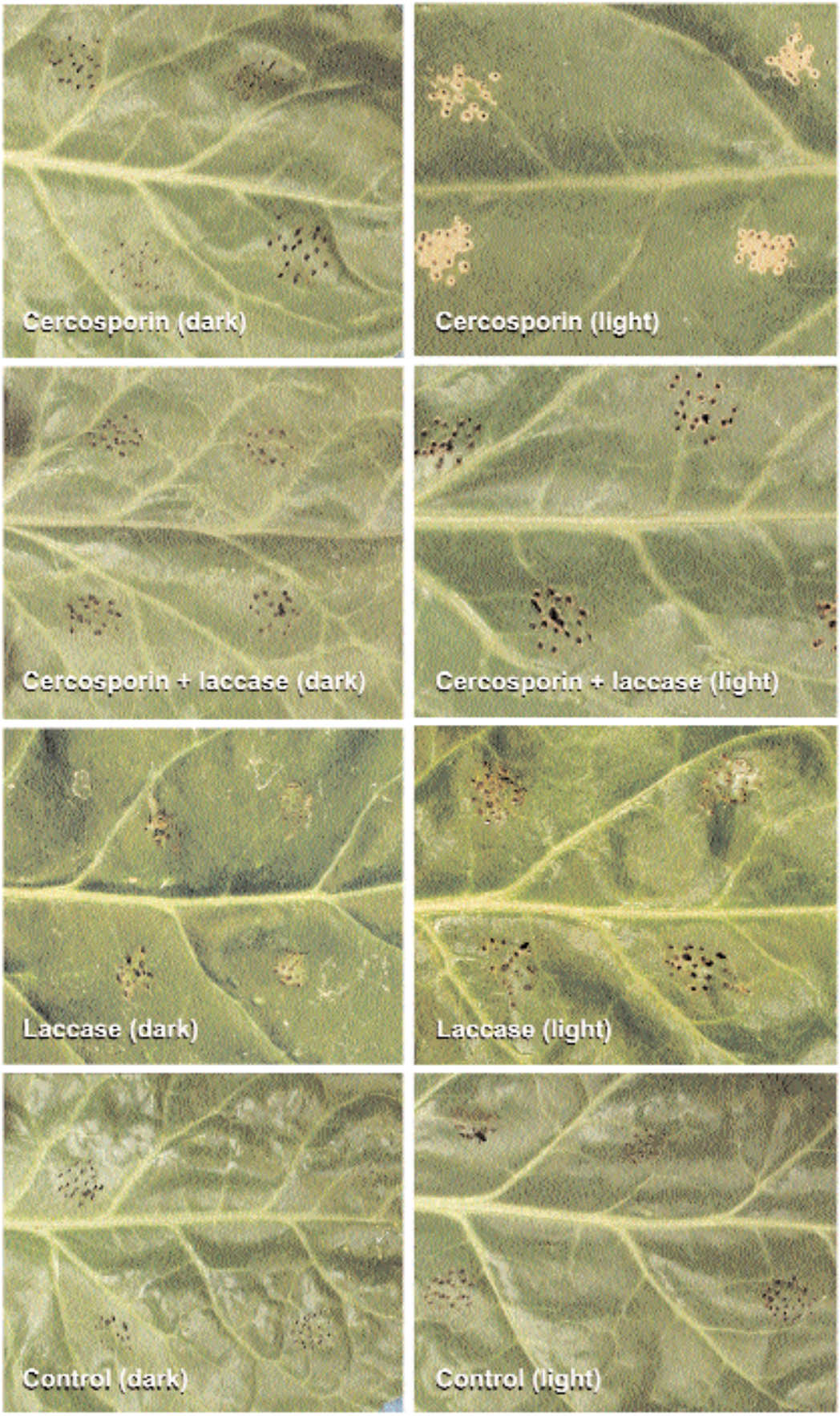

Fig. 4. Necrotic symptoms of fresh sugar beet leaf surfaces induced by cercosporin (100 …g ml^{-1}) from *C. beticola* (C2) and/or laccase (100U ml^{-1}) from *P. ostreatus*. Incubation was for 48 h at 25°C in the dark or under constant light.

(data not shown) and decrease the absorbance of the pigment Poly B-411 (Fig. 2). After 5 min of reaction, a more drastic decrease in the absorbance of Poly B-411 was found in preparation with *P. ostreatus* laccase (OD 2.47 ± 0.09) than with *T. versicolor* laccase (OD 5.63 ± 0.12) when compared to the control without laccase added (OD 6.4 ± 0.10). No significant changes in the enzyme activity were found after the first 5 min. These results indicating that both laccases have oxidative activity potential are in accordance with Platt *et al.* (1985) who have demonstrated that *P. ostreatus* laccase can oxidize the pigment Poly B-411 in both liquid and solid medium and with Wong and Yu (1999) who have showed that *T. versicolor* laccase can degrade synthetic dyes with typical chromophores (anthraquinone, azo and indigo).

The UV-visible absorption spectra of cercosporin extracted from either *C. beticola* C2 or *C. hayi* in the course of incubation with *P. ostreatus* or *T. versicolor* laccase in the dark were investigated in this study. Figure 3 illustrates the UV-visible absorption spectrum of *C. beticola* C2 cercosporin. The maximum absorption of cercosporin from the two sources, at 220, 279, and 495-500 nm, was in good agreement with spectra reported previously by Yamazaki and Ogawa (1972) in their study of the chemistry and stereochemistry of cercosporin from *Cercospora kikuchii* Matsumoto & Tomoy. Cercosporin from C2 and *C. hayi* (data not shown) presented similar visible decreases in absorbance at 220, 279 and 495-500 nm within 10 min of reaction with *P. ostreatus* or *T. versicolor* laccase compared to the controls without laccase added. For example, cercosporin from C2 showed decreases in absorbance of 48.61%, 47.97%, and 53.13% at 220, 279, and 500 nm respectively with *P. ostreatus* laccase, and 35.50%, 35.09%, and 41.79 % with *T. versicolor* laccase, and a peak shift of 21 nm at 500 nm. A gradual decolorization of cercosporin was noted from deep red to light pink for both sources of cercosporin during the enzymatic reaction. These results indicate that laccase from basidiomycetes can degrade *C. beticola* and *C. hayi* cercosporin. Since the enzymatic reaction was performed in the dark where no reactive oxygen species are generated, we can assume that the degradation of cercosporin was due only to the activity of the basidiomycete laccase.

Although a direct comparison cannot be made, these results indicate that both *P. ostreatus* and *T. versicolor* laccases react not only with a perylenequinone pigment (cercosporin) but also with an anthraquinone-based pigment (Poly B-411). Edens *et al.* (1999) have shown that laccase from *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier, a fungus causing take-all disease in wheat and barley can decolorize Poly B-411, an effect suggesting a role in lignin degradation. Anthraquinone is an organic compound whose structure serves as a basic building block for a number of naturally occurring pigments. Interestingly, other pigments produced by *C. beticola* are polymeric anthraquinone-based pigments, such as cebelin (Jalal *et al.,* 1992) and beticolin (Goudet *et al.*, 2000) which cause a broad range of cytotoxic effects on plants and animals similar to cercosporin. It would be informative to investigate whether or not ligninolytic enzymes, including laccases and peroxidases can affect the toxicity of the anthraquinone-based pigments produced by *C. beticola*.

Bacteria and plant tissue response to cercosporin and laccase. Table 1 shows the effects of cercosporin from isolate C2) and/or laccase from *P. ostreatus* or *T. versicolor* on bacterial cells after incubation for 1, 2 and 3 h in the dark and under constant light. The percentage of the total number of viable cells between treatments was not significantly different in the dark but was significantly the lowest when cells were exposed to cercosporin for 1, 2, and 3 h under constant light. For example after 1, 2, and 3 h under light, the number of viable cells was respectively 38.79%, 54.44% and 53.28% in cercosporin treatment and 97.06%, 97.67% and 97.22% in the controls without cercosporin added. This indicates that cercosporin was toxic to *E. coli* bacteria under constant light. This is probably due to the production of toxic oxygen radicals by cercosporin known to cause oxidation of membrane fatty acids, proteins, carbohydrates and nucleic acids leading to cell death. However, the number of viable cells exposed under light to the mixture of cercosporin and laccase was significantly higher than when cells were exposed to cercosporin alone. For example, after 1 h under light, the number of viable cells was 89.07% in the cercosporin-laccase treatment, compared to 38.79 % in the treatment with cercosporin alone. This suggests that laccase from *P. ostreatus* basidiomycete reduced the toxicity of cercosporin extracted from C2 isolate to *E. coli*.

Figure 4 shows the necrotic symptoms of sugar beet leaves induced by *C. beticola* isolate C2 cercosporin and/or *P. ostreatus* laccase. After 48 h of incubation in the dark, no striking difference in the necrosis of the treated areas was detected compared to the controls, except that in the controls the dark spots observed in all treatments were caused by cell injury during delivery of the compounds using the tip of a sharp needle. However, incubation under constant light of cercosporin-treated samples showed dark spots surrounded by circular to irregular yellow-brown margins which appear dry and crusty. These necrotic lesions become enlarged and occasionally extended to the whole treated area at the end of the incubation period. However, when samples were treated with the cercosporin-laccase mixture, necrotic zones were greatly reduced, compared to the cercosporin-treated samples. No lesions were found on leaf surfaces exposed to laccase alone or in the controls under light. After 48h of incubation under light, the rank

of the data based on visual basis was cercosporin > cercosporin-laccase mixture > laccase, control (Table 2). This suggested that the toxicity of cercosporin toward sugar beet leaf tissue can be reduced by basidiomycete laccase. The damaging effect of extracted cercosporin on sugar beet leaves was previously reported (Steinkamp *et al.,* 1981) using electron microscopy to describe the disruption of the plasmalemma, tonoplast, and chloroplast membranes. More recently a similar study involved tobacco leaves (Panagiotis *et al.*, 2007).

Studies have indicated that soil is a source of primary inocula of leaf spot disease of sugar beet (Nagel, 1938; Giannopolitis, 1978). Crop debris has been demonstrated to serve as a source of inoculum of *C. beticola* for at least two years (Jones and Windels, 1991; McKay and Pool, 1918). A recent 3-yr study (Khan *et al.*, 2008) reported that inocula of *C. beticola* as infested beet residue survived longest (22 months) on the soil surface, with inocula buried 10 or 20 cm deep surviving for 10 months. Thus, if the infested debris is incorporated, the chances of the pathogen to survive are decreased but such debris remaining on the soil surface would serve as the source of primary inoculum. To target the primary inoculum, we thus propose the use of an enzymatic approach to diminish the chances of the pathogen to persist in leaf debris. Since saprophytic basidiomycete fungi which produce large amount of lignolytic enzymes, such as laccases, peroxidases, and lignin peroxidases, are important decomposers of crop residue (Thorn *et al.*, 1996; Stubbs *et al.*, 2004), the use of laccase-producing basidiomycete fungi as antagonists to control *C. beticola* in soil is an approach which we are currently investigating. The question arises as to whether adding laccase as an active catalyst on *C. beticola*-infested plant debris could contribute to ultimately decreasing the inoculum of the pathogen in soil. Considering that cercosporin helps the pathogen to protect itself against antagonists on plant tissue, if the mode of action of laccase on cercosporin can attenuate the defense mechanisms of the pathogen, this might offer a new approach for the development of supervised control systems. Surface-borne inoculum could thus be the target of laccases that could both degrade cercosporin being produced for antibiosis by stromata on the soil surface against antagonists and decompose beet leaf tissue bearing stromata. Immunological methods could be used to detect and quantify *C. beticola* in natural soil to monitor its inoculum (Caesar-TonThat *et al.,* 2007). Extracellular enzymes such as laccase are inactivated by absorption (particularly to clay), denatured by chemical factors in soil (e.g., pH, ionic composition of soil solution), or serve as substrates for proteolytic microorganisms. To improve the chance of laccase to persist long enough to degrade cercosporin, laccase activity could be improved and stabilized by immobilization of the enzyme (Leonowicz *et al.,* 1988; Palmieri *et al.*, 1994; Sarkar *et al.*, 1989) on solid supports (e.g. kaolinite) to increase its resistance to degradation by proteases and protect it against changes in pH, temperature, or fluctuations of ionic strength. However, large-scale production of laccase may not be economically feasible, and thus laccase-producing basidiomycetes adapted to the soils involved may be the best approach.

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REFERENCES

Abadulla E., Tzanov T., Costa S., Robra K.H., Cavaco P.A., Guebitz G.M., 2000. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Applied Environmental Microbiology* **66**: 3357-3362.

Bourbonnais R., Paice M.G., Reid I.D., Lanthier P., Yaguchi M., 1995. Lignin oxidation by laccase isoenzyme from *Trametes versicolor* and role of the mediator 2,2'-azino bis (3-ethylbenzthiazoline-6-sulphonic acid) in kraft lignin depolymerisation. *Applied Environmental Microbiology* **61**: 1876-1880.

Caesar-TonThat T.C., Lartey R.T., Shelver W.L., 2007. Enzyme-linked immunosorbent assay for *Cercospora beticola* in soil. *Journal of Sugarbeet Research* **44**: 51-70

Cho N.-S, Kim D.-H., Cho H.-Y., Ohga S., Leonowicz A., 2006. Effect of various compounds on the activity of laccases from basidiomycetes and their oxidative and demethoxylating activities. *Journal of the Faculty of Agriculture of Kyushu University* **51**: 211-218.

Claus H., Faber G., König H., 2002. Redox-mediated decolorization of synthetic dyes by fungal laccases. *Applied Microbiology and Biotechnology* **59**: 672-678.

Claus H., 2004. Laccases:structure, reactions, distribution. *Micron* **35**: 93-96.

Daub M.E., 1982. Cercosporin, a photosentitizing toxin from *Cercospora* species. *Phytopathology* **72**: 370-374.

Daub M.E., Hangarter, R.P., 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. *Plant Physiology* **73**: 855-857.

Daub M.E., Leisman G.B., Clark R.A., Bowden E.F., 1992. Reductive detoxification as a mechanism of fungal resistance to singlet oxygen-generating photosensitizers. *The Proceedings of the National Academy of Sciences USA* **89**: 9588-9592.

Daub M.E., Ehrenshaft M., 2000. The photoactivated *Cercospora* toxin cercosporin: contributions to plant disease and fundamental biology. *Annual Review of Phytopathology* **38**: 437-466.

Durán N., Esposito E., 2000. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wasterwater and soil treatment: a review. *Applied Catalysis B: Environmental* **28**: 83-99.

Edens W.A., Goins T., Dooley D., Henson J.M., 1999. Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritici*. *Applied Environmental Microbiology* **65**: 3071-3074.

Ferrari R.P., Laurenti E., Ghibaudi R.M., Casella L., 1997. Tyrosinase-catecholic substrates *in vitro* model: kinetic studies on the oquinone/o-semiquinone radical formation. *Journal of Inorganic Chemistry* **68**: 61-69.

Fukuda T., Uchida H., Takashima Y., Uwajima T., Kawabata T., Suzuki M., 2001. Degradation of bisphenol A by purified laccase from *Trametes villsoa*. *Biochemical and Biophysical Research Communications* **284**: 704-706.

Giannopolitis C.N., 1978. Lesions on sugarbeet roots caused by *Cercospora beticola*. *Plant Disease Reporter* **62**: 424-427.

Gold M.H., Glenn J.K., Alic M., 1988. Use of polymeric dyes in lignin biodegradation assays. *Methods of Enzymology B* **161**: 74-78.

Goudet C., Milat M.-L., Sentenac H., Thibaud J.-B., 2000. Beticolins, non peptidic, polycyclic molecules produced by the phytopathogenic fungus *Cercospora beticola*, as a new family of ion channel-forming toxins. *Molecular Plant-Microbe Interactions* **13**: 203-209.

Hartman P.E., Dixon W.J., Dahl T.A., Daub M.E., 1988. Multiple modes of photodynamic action by cercosporin. *Photochemistry and Photobiology* **47**: 699-703.

Hofrichter M., Fritche W., 1997. Depolymerization of low-rank coal by extracellular fungal enzyme systems II. The ligninolytic enzymes of the coal-humic-acid-depolymerizing fungus *Nematoloma frowardii* b19. *Applied Microbiology and Biotechnology* **47**: 419-424.

Jalal M.A.F., Hossain M.B., Robeson D.J., van der Helm D., 1992. *Cercospora beticola* phytotoxins: Cebetins that are photoactive, Mg2+-binding, chlorinated anthraquinone-xanthone conjugates. *Journal of the American Chemical Society* **114**: 5967-5971.

Jaszek M., Grzywnowicz K., Malarczyk E., Leonowicz A., 2006. Enhanced extracellular laccase activity as a part of the response system of white rot fungi: *Trametes versicolor* and *Abortiporus biennis* to paraquat-caused oxidative stress conditions. *Pesticide Biochemistry and Physiology* **85**: 147-154.

Jenns A.E., Scott D.L., Bowden E.F., Daub M.E., 1995. Isolation of mutant of the fungus *Cercospora nicotianae* altered in their response to singlet oxygen-generating photosensitizers. *Photochemistry and Photobiology* **61**: 488-493.

Jones R.K., Windels C.E., 1991. A management model for Cercospora leaf spot of sugarbeets. *University of Minnesota Extension Service* AG-FO-5643-E, Twin Cities, MN, USA.

Khan J., del Rio L.E., Nelson R., Rivera-Varas V., Secor G.A., Khan M.F.R., 2008. Survival, dispersal, and primary infection site for *Cercospora beticola* in sugar beet. *Plant Disease* **92**: 741-745.

Kuyama S., Tamura T., 1957. Cercosporin. A pigment of *Cercospora kikuchii* Matsomuto et Tomoyasu. I. Cultivation of fungus, isolation and purification of pigment. *Journal of the American Chemical Society* **79**: 5725-5726.

Leonowicz A., Trojanowski J., Barbara O., 1979. *Basidiomycetes*: apparent activity of the inducible and constitutive forms of laccase with phenolic substrates. *Acta Biochimica Polonica* **25**: 369-378.

Leonowicz, A., Sarkar J.M., Bollag J.-M., 1988. Improvement in stability of an immobilized fungal laccase. *Applied Microbiology and Biotechnology* **29**: 129-135.

Lynch F.J., Geoghegan M.J., 1979. Antibiotic activity of a fungal perylene-quinone and some of its derivatives. *Transactions British Mycological Society* **72**: 31-37.

Macri F., Vianello A., 1979. Inhibition of K^+ uptake, H^+ extrusion and K^+-activated ATPase, and depolarization of transmembrane potential in plant tissues treated with *Cercospora beticola* toxin. *Physiological Plant Pathology* **15**: 161-170.

Mayer A.M., Staples R.C., Gil-ada N.L., 2001. Mechanisms of survival of necrotrophic fungal plant pathogens in hosts expressing the hypersensitive response. *Phytochemistry* **58**: 33-41.

McKay M.B., Pool V.W., 1918. Field studies of *Cercospora beticola*. *Phytopathology* **8**: 119-136.

Nagel C.M., 1938. The longevity of *Cercospora beticola* in soil. *Phytopathology* **28**: 342-350.

Palmieri G., Giardina P., Desiderio B., Marzullo L., Giamberini M., Sannia G., 1994. A new enzyme immobilization procedure using copper alginate gel: application to a fungal phenol oxydase. *Enzyme and Microbial Technology* **16**: 151-158.

Panagiotis M., Kritonas K., Obeidat Irini N., Kiriaki C., Nicolaos P., Athanasios T., 2007. Expression of the yeast cpd1 gene in tobacco confers resistance to the fungal toxin cercosporin. *Biomolecular Engineering* **24**: 245-251.

Platt M.W., Hadar Y., Chet L., 1985. The decolorization of the polymeric dye Poly-Blue (polyvinalamine

sulfonate-anthroquinone) by lignin degrading fungi. *Applied Microbiology and Biotechnology* **21**:394-396.

Rama R., Mougin C., Boyer F.-D., Kollmann A., Malosse C., Sigoillot J.-C., 1998. Biotransformation of benzo[a]pyrene in bench scale reacter using laccase of *Pycnoporus cinnabrinus*. *Biotechnology Letters* **20**: 1101-1104.

Rodriguez E., Pickard M.A., Vasquez-Duhalt R., 1999. Industrial dye decolorization by laccases from ligninolytic fungi. *Current Microbiology* **38**: 27-32.

Sarkar, J.-M., Leoniwicz A., Bollag J.M., 1989. Immobilization of enzyme on clays and soils. *Soil Biology and Biochemistry* **21**: 222-230.

Steinkamp M.P., Martin S.S., Hoefert I.I., Ruppel E.G., 1981. Ultrastructure of lesions produced in leaves of *Beta vulgaris* by cercosporin, a toxin from *Cercospora beticola*. *Phytopathology* **71**: 1272-1281.

Stubbs T.L., Kennedy A.C., Schillinger W.F., 2004. Soil ecosystem changes during the transition to no-till cropping. *Journal of Crop Improvement* **11**: 105-135.

Thorn R.J., Reddy C.A., Harris D., Paul E.A., 1996. Isolation of saprophytic basidiomycetes from soil. *Applied Environmental Microbiology* **62**: 4288-4292.

Taylor T.V., Mitchell T.K., Daub M.E., 2006. An oxidoreductase is involved in cercosporin degradation by the bacterium *Xanthomonas campestris* pv. Zinniae. *Applied Environmental Microbiology* **72**: 6070-6078.

Thurston C.F., 1994. The structure and function of fungal laccases. *Microbiology* **140**: 19-26.

Ververidis P., Davrazou F., Diallinas G., Georgakopoulos D., Kanellis A.K., Panopoulos N., 2001. A novel putative reductase (Cpd1p) and the multidrug exporter Snq2p are involved in resistance to cercosporin and other singlet oxygen-generating photosensitizers in *Saccharomyces cerevisiae*. *Current Genetics* **39**: 127-136.

Whitney E.D., Lewellen R.T., 1976. Identification and distribution of races of C1 and C2 of *Cercospora beticola* from sugar beet. *Phytopathology* **66**: 1158-1160.

Ward J.M.J., Stromberg E.L., Nowell D.C., Nutter J.F.W., 1999. Gray leaf spot: a disease of global importance in maize production. *Plant Disease* **83**: 884-895.

Windels C.E., Lamey H.A., Hilde D., Widner J., Knudsen T., 1998. A *Cercospora* leaf spot model for sugar beet. In practice by an industry. *Plant Disease* **82**: 716-726.

Wong Y., Yu J., 1999. Laccase-catalyzed decolorization of synthetic dyes. *Water Research* **33**: 3512-3520.

Yamazaki S., Okubo A., Akiyama Y., Fuwa K., 1975. Cercosporin, a novel photodynamic pigment isolated from *Cercospora kikuchii*. *Agricultural Biology and Chemistry* **39**: 287-288.

Yamazaki S., Ogawa T., 1972. The chemistry of cercosporin. *Agricultural Biology and Chemistry* **36**: 1707-1718.

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